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FINAL REPORT

Neurotoxic Properties of Endotoxins

by

Robert J. Person, Ph.D.



University of Oklahoma Health Sciences Center
Department of Physiology and Biophysics
Oklahoma City, Oklahoma 73190

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ABSTRACT

Some endotoxins produce a biphasic effect on spontaneous transmitter secretion, with an early facilitation of release, but a monotonic depression of evoked release triggered by nerve stimulation, while lipopolysaccharide produces only a monotonic depression of both spontaneous and evoked transmitter secretion under physiological conditions. However, glycolipid produces a biphasic effect on both spontaneous and evoked release. This suggests that the variety of effects seen in less pure preparations of glycolipids are modulations of the biphasic pattern of action resulting from differences in extraction procedures, presence of polysaccharide, or contamination of the specific endotoxin preparation employed. Based on these experiments we have proposed experimentally based mechanisms for the action of bacterial glycolipid: (1) an early, transient ionophoric action for calcium which facilitates secretion and (2) a late increasing isolation of the membrane from extracellular space.

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Previous work has described significant neurotoxic action of endotoxins (ETX) derived from E. Coli and Salmonella. These effects are, generally, to block the release of transmitter from presynaptic terminals, thus incapacitating peripheral neural transmission. Our earliest hypothesis for the cause of this blockade was that ETX first increases calcium permeability across the presynaptic terminal membrane, by possibly serving as a calcium ionophore, then decreases calcium permeability resulting in abolition of evoked transmitter release and a drastic reduction abolition of spontaneous transmitter release.

Later work (J. Neuroscience Research, 4:105, 1979) suggested that the abolition of transmitter release might be effected by a more general mechanism. Endotoxin may isolate the presynaptic terminal from the extracellular environment and, as a consequence, prevent calcium influx when required for transmitter release. A reasonable hypothesis would then be that ETX blocks transmitter release by virtue of the extreme lipophilic character of the lipidA moiety but may cause an early transient rise in transmitter release by an, as yet, unknown fraction of the crude macromolecule. If this hypothesis is correct, we could perhaps explain the vasodilatory effects of ETX in systemic shock simply by invoking the same mechanism for smooth muscle: vasoconstrictor tone would be lost as the muscle membrane becomes isolated from calcium in the extracellular fluid.

We demonstrated subsequently that the biphasic pattern of ETX action on spontaneous transmitter secretion could also be observed in the presence of heat-killed Salmonella (Experientia, 35:801, 1979) and that membrane isolation, as demonstrated by blockade of the cation ionophore X537 probe also occurred with time. Testing of a variety of ETX preparations, including additional materials derived from TCA extraction (Boivin method) and material produced via phenol extraction demonstrated clear differences in ETX potency as a function of extraction procedure and purity. We were thus able to suggest that the variety of experimental findings of other investigators are at least partially explained by contamination and impurity of the normally used ETX preparations. In fact, we have stated that comparison of experiments using non-standardized preparations of ETX is impossible and many data produced by investigators using such preparations at ETX will be impossible to reproduce. In keeping with the diversity of preparations used a standard nomenclature should be defined. The nomenclature in the discussion below will be as follows:

1. Endotoxin (ETX) Generic for gram negative bacterial cell wall products regardless of source, extraction procedure, or purity
2. Crude ETX (CETX) Indicates generally used endotoxin regardless of extraction procedure, contaminated by protein, and of unpredictable effect on the neural membrane
3. CETX/phenol or Crude ETX indicating extraction procedure using trichloroacetic acid (Boivin procedure) or phenol (Westphal procedure)

4. Lipopolysaccharide (LPS)	Tested as RNA-Free, pure ETX in some references, without regard to bacterial source, may be either <u>Salmonella</u> or <u>E. Coli</u> derived in present work
5. LPS/S or LPS/E	LPS from <u>Salmonella</u> , or LPS from <u>E. Coli</u>
6. Glycolipid (LPA)	Derived from <u>S. Minnesota</u> , Re-595, no polysac- caride present, used as analogue for Lipid A in present work

MORE RECENT RESULTS INCLUDES THE FOLLOWING:

A. Toxicity of S. Typhimurium, heat-killed. We concluded that biphasic activity and membrane isolation components of ETX were present. The time courses of these effects are extremely long and indicate a prolonged sequence of events in the bacterial membrane and neural membrane interaction. If these effects are, in fact, due to LPA interaction with the membrane than components of the Salmonella membrane at this state of purification slow the LPA action but cannot prevent it.

B. Toxicity of ETX/TCA. After demonstrating the biphasic pattern of some ETX/TCA preparations, of heat-killed Salmonella, and the monotonic pattern of LPS/S we returned to ETX/TCA and attempted to replicate the biphasic pattern observed previously. As shown in Figure 1 a sample of ETX/TCA was ineffective in altering secretion rate while ETX/P produced only a monotonic depression of secretion rate. For comparison, Figure 1 shows also the pattern of depression produced by LPS/S. This comparison as indicative of the unpredictability of ETX/TCA in this system and the relatively clean effect of ETX/P, comparable to LPS, albeit with no biphasic pattern. We sought some biological activity in the debris left from the ETX/P extraction but found no significant effects. At this point we were faced with the paradox of a facilitation in secretion occurring with heat-killed Salmonella and with some ETX/TCA, but not in response to samples of ETX/P, LPS/S, or LPS/E. The results of these experiments suggest the futility of basing any explanation of the biological effects of ETX on experiments using ETX/TCA.

C. Toxicity of glycolipid from Re-595, S. Minnesota. These results are preliminary but the implications of the data collected thus far are clear. The most important observation made is that both spontaneous and evoked secretion of transmitter release undergo significant facilitation prior to depression. Figure 2 shows averaged results from 9 experiments in which MEPP was observed using 10 µg LPA/ml: a biphasic pattern is clear but variability is high. The variability derives principally from the range of latencies of peak transmitter (MEPP) release rates. Figure 3 shows individual experiments over a range of 10 µg to 1 mg LPA/ml bath. Two effects are apparent: (1) transmitter release is either increased or depressed slowly as a function of dose down to a concentration of 10ng/ml, (2) at higher doses (10 and 1 µg/ml) there is a second component apparent which is marked by abrupt decrease (faster decline) in the slope of MEPP rate depression with a final stabilization. With decreasing concentrations the late phase disappears.

Figure 4 shows the effects of LPA on evoked transmitter release (measured by the amplitude of intracellularly recorded endplate potentials) over a concentration range of 10 μg to 1 ng LPA/ml bath. Again two components of action are immediately apparent: (1) a slow component of either facilitation or slight depression of evoked transmitter release and (2) a component of depression with more negative (faster decline) slope. At the highest concentration (10 $\mu\text{g}/\text{ml}$) endplate potentials are elevated, indicating an ionophoric effect of LPA, at 80 min of exposure there is an abrupt decline in amplitude which continues to complete blockade within a few minutes. With lower concentrations the first component is converted to a depression of faster than slower decline while the second component is converted to shallower slope and the appearance of a final baseline. These data suggest that the initial, calcium ionophoric component of LPA action serves to keep transmitter secretion elevated; at some point in time subsequent to the beginning of exposure of LPA the second component predominates and the membrane becomes more or less isolated from external calcium. The kinetics of experiments at 1 and 10 $\mu\text{g}/\text{ml}$ suggest that there can be a sudden transition from one component to the second with in a few minutes. In some experiments the transition may be so swift that first component is either absent or it cannot be resolved at sensitive junctions.

Figure 5 describes a single experiment in which LPA and calcium are shown to be simple antagonists. The junction shown was depressed immediately by 1 $\mu\text{g}/\text{ml}$ LPA, depression was not reversed by return to LPA-free control bath but was immediately reversed to control with the addition of 0.5 mM calcium. With reintroduction of 1 $\mu\text{g}/\text{LPA}/\text{ml}$ bath in the presence of elevated calcium (now 2.5 mM) transmitter release is facilitated above control. Subsequently, when LPA concentration is raised to 10 $\mu\text{g}/\text{ml}$ bath, depression occurs immediately, stabilizes, and can then be reversed by a large elevation in bath calcium (to 16.6 mM in this experiment). This and similar experiments suggest a clear antagonism between calcium and LPA during the early component of LPA action. The measurement of quantal content (right-hand coordinate) using the method of failures is an indication that the reduction in EPP amplitude is presynaptic in origin. There are however, suggestions in this (e.g., at 15 to 25 min) and in other experiments that LPA may have a significant postsynaptic action.

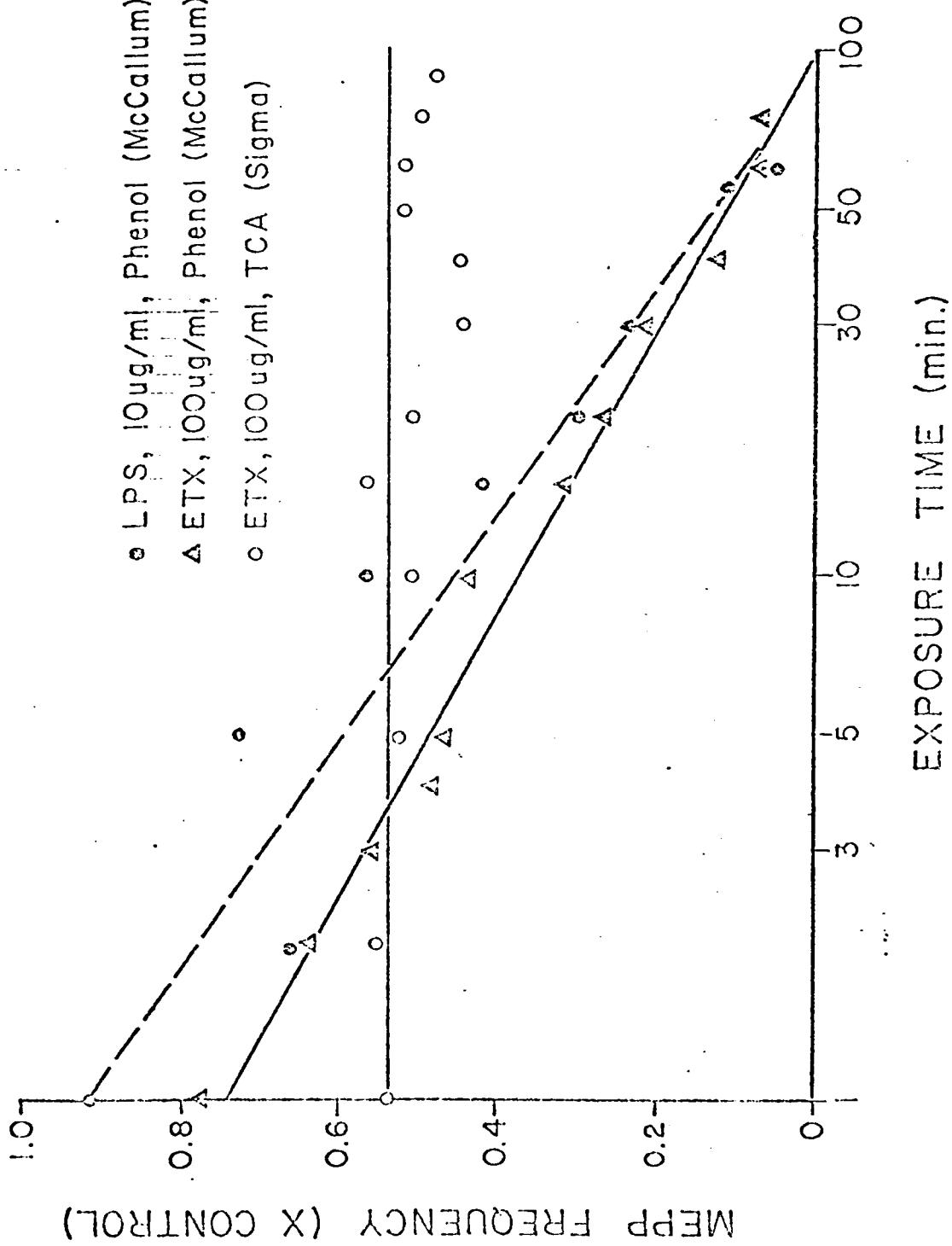
Assuming that preliminary work showing a biphasic action of LPA on both spontaneous and on evoked secretion of transmitter is confirmed in additional experiments, we suggest that a working hypothesis of ETX action on the neural membrane can be proposed: The interaction of ETX and the membrane consists of at least two stages or components. The first component (Component I) involves an increase in calcium permeability across the membrane. This component is transient, subject to considerable variability in potency as a function of level of purification, purification procedure (e.g., TCA vs. phenol), and contamination. The second component (Component II) it involves the inevitable and possibly irreversible isolation of the membrane from extracellular space.

Component I is expressed in our system as (1) an increase in the rate of spontaneous transmitter release which apparently derives from an increase in resting calcium permeability and (2) as an increase in the total "activated" calcium permeability induced by membrane depolarization which results in a facilitation of evoked transmitter release. In other systems Component I should be expressed as increased levels of spontaneous

or evoked hormone release, increased tone in smooth muscle and increased contractility in both smooth and cardiac muscle as a function of their respective complete and partial dependence on extracellular calcium, and increased autonomic drive due to facilitation of ganglionic and end organ neurotransmission.

Component II is expressed in our system as a decreased rate of spontaneous transmitter release, as a depression or blockade of evoked transmitter release, by the inability of potent cation ionophores to provoke increased transmitter release or restore it after total blockade, the inability of increased extracellular calcium to increase or restore transmitter release. Component II should be expressed in other systems as decreased hormone secretion to previously adequate physiological stimuli, decreased tone in spontaneously active smooth muscle, decreased contractility in smooth and cardiac muscle, (again as a function extracellular calcium requirements) a depression of autonomic reflexes, and generally as a debilitation of all peripheral calcium dependent processes.

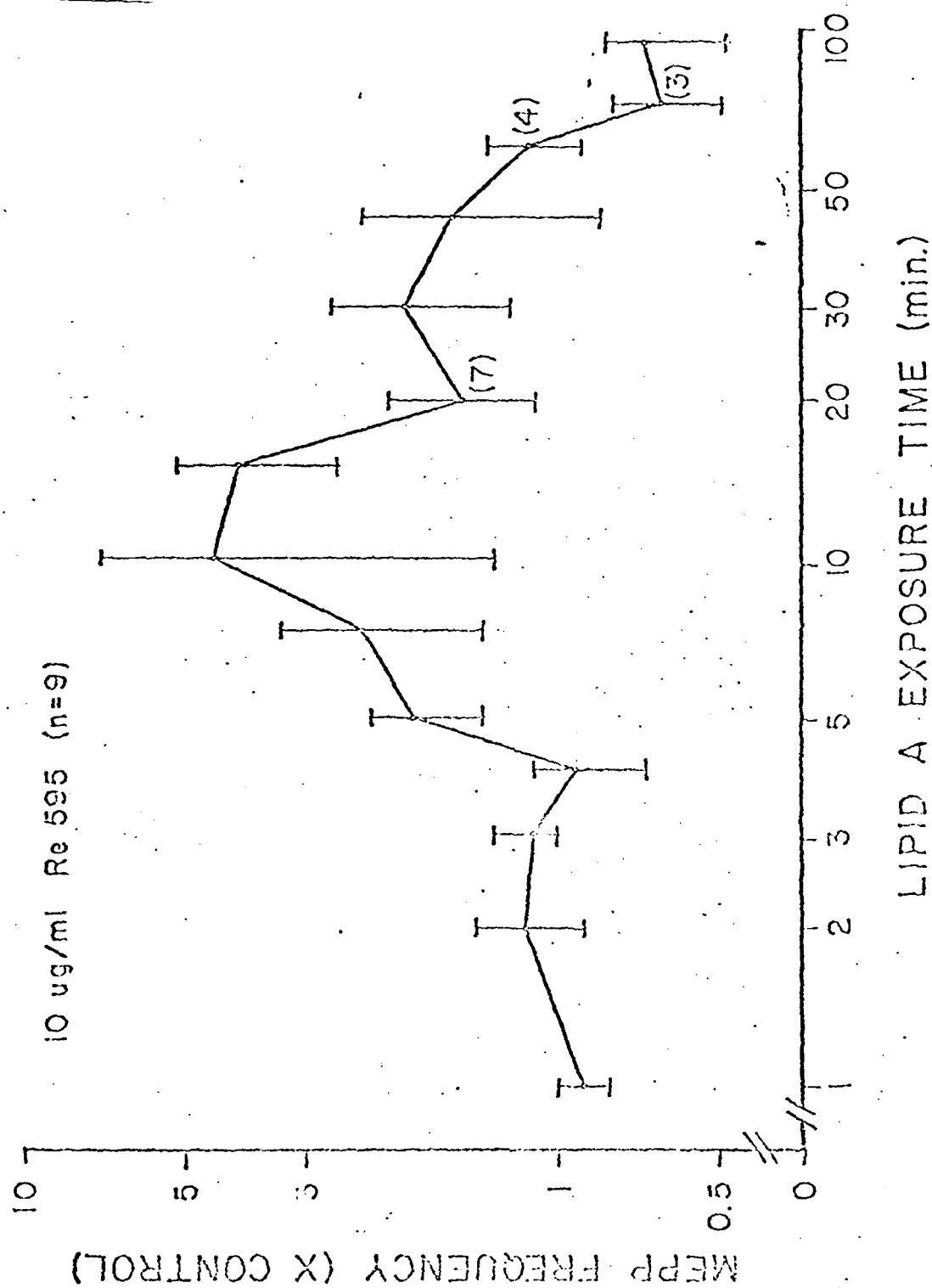
A schematic portrayal of the dual component mode of ETX action is shown in Figure 6. It should be emphasized that the specification of two components in the action of ETX may well be a convenient fiction but it serves as a good working hypothesis. It is conceivable that the two components are expressions of a continuous process of rearrangement of the neural membrane during a prolonged interaction with ETX, the eventual endpoint of which is membrane isolation. Some of the data we have developed suggests that LPA may attach at or near the membrane site of the normally present "slow calcium channel" ordinarily activated by membrane depolarization. The act of LPA attachment could first distort the channel, transiently increasing calcium permeability, while at the same time a process of membrane isolation, transformation, substitution, or isolation is initiated which eventually reduces calcium permeability to nil.

Figure 1

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FIGURE 2



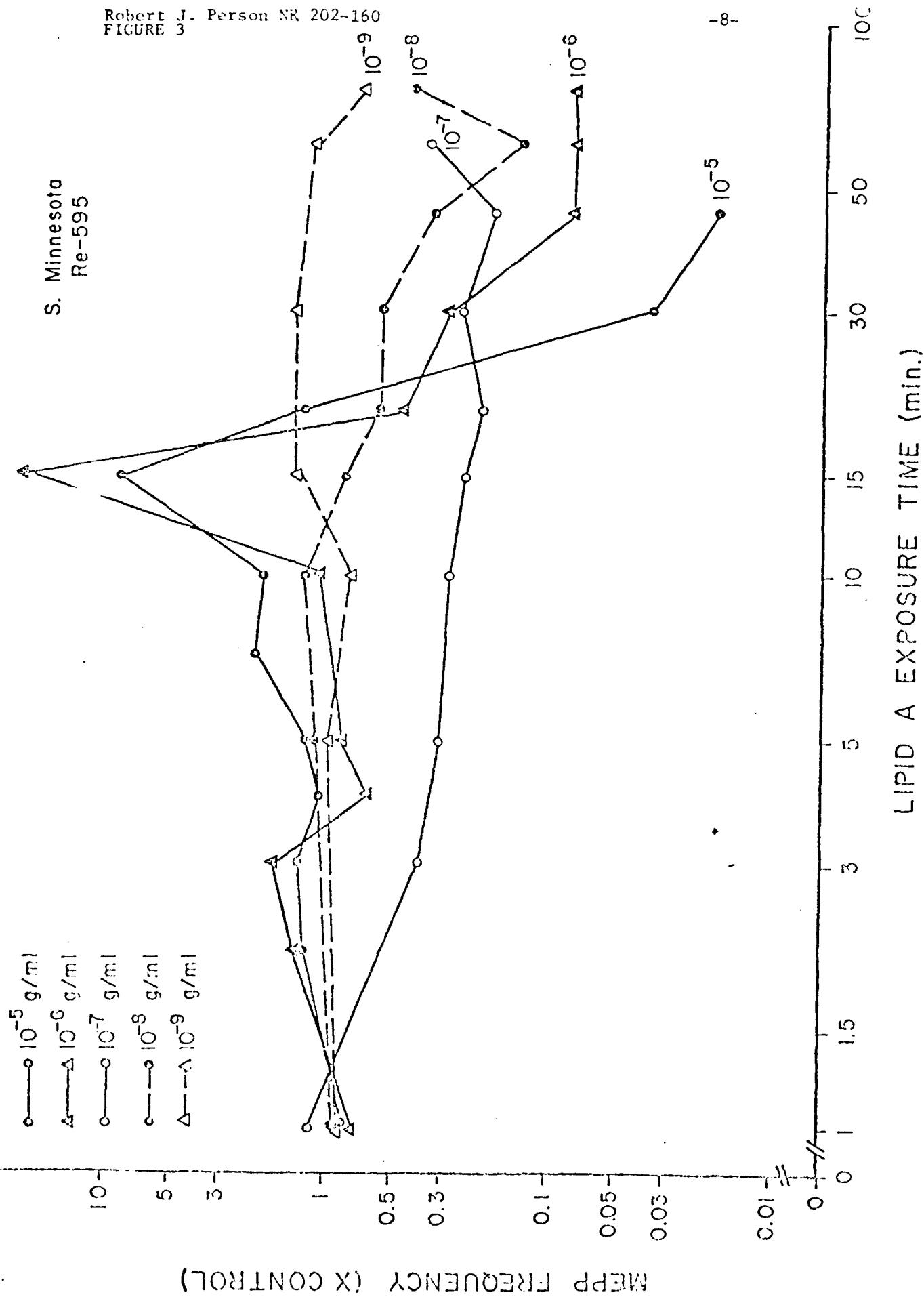


Figure 4

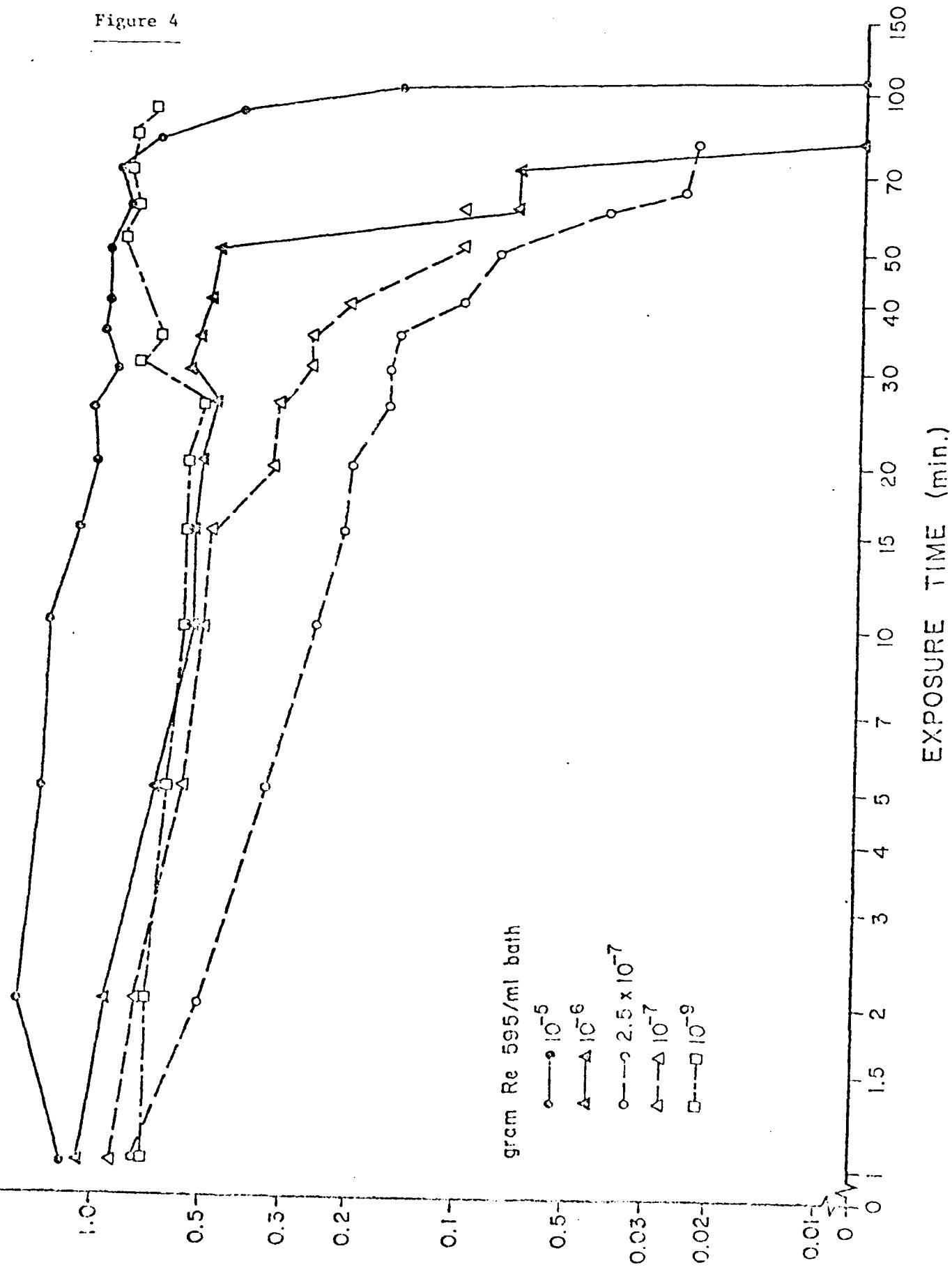


FIGURE 5

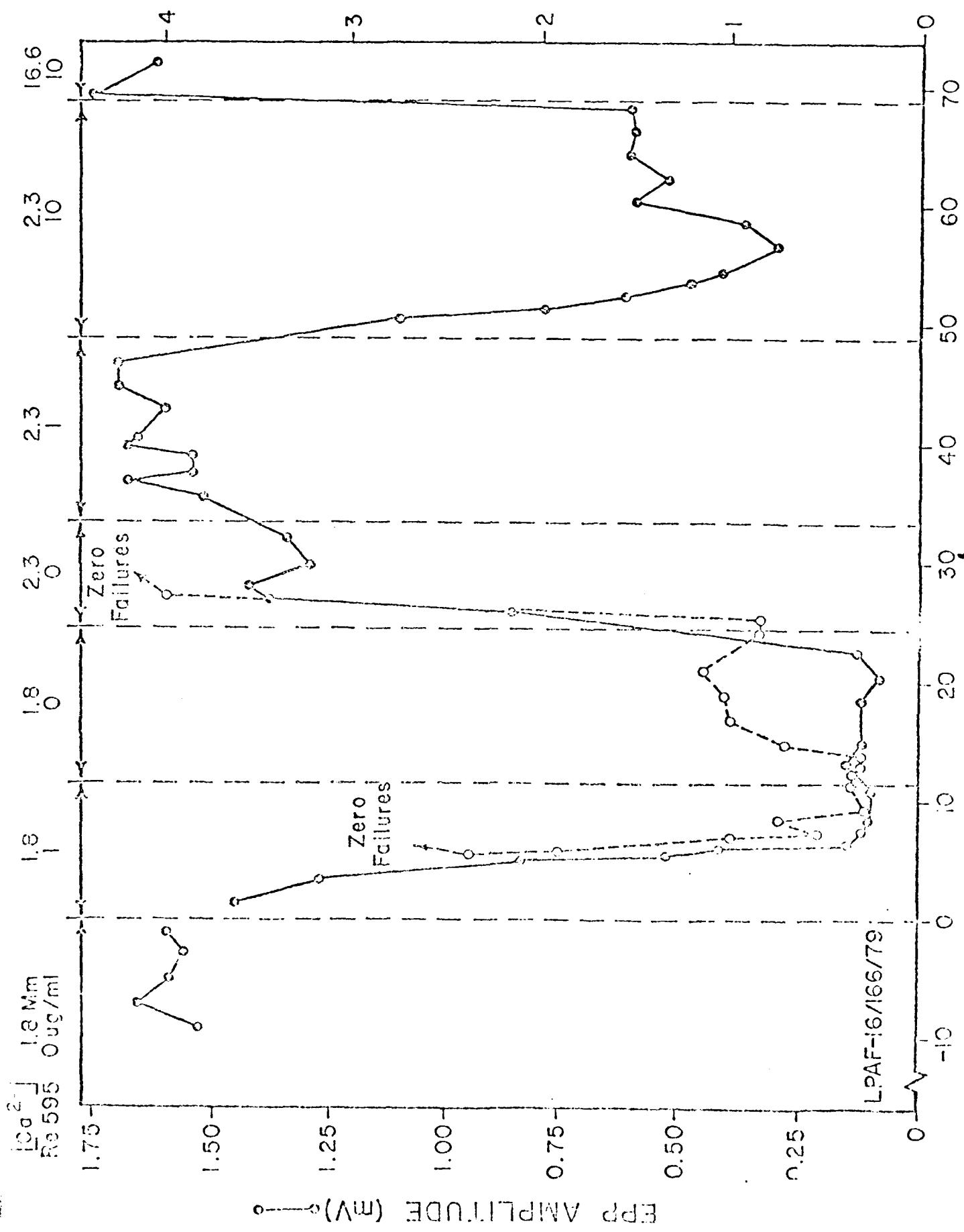
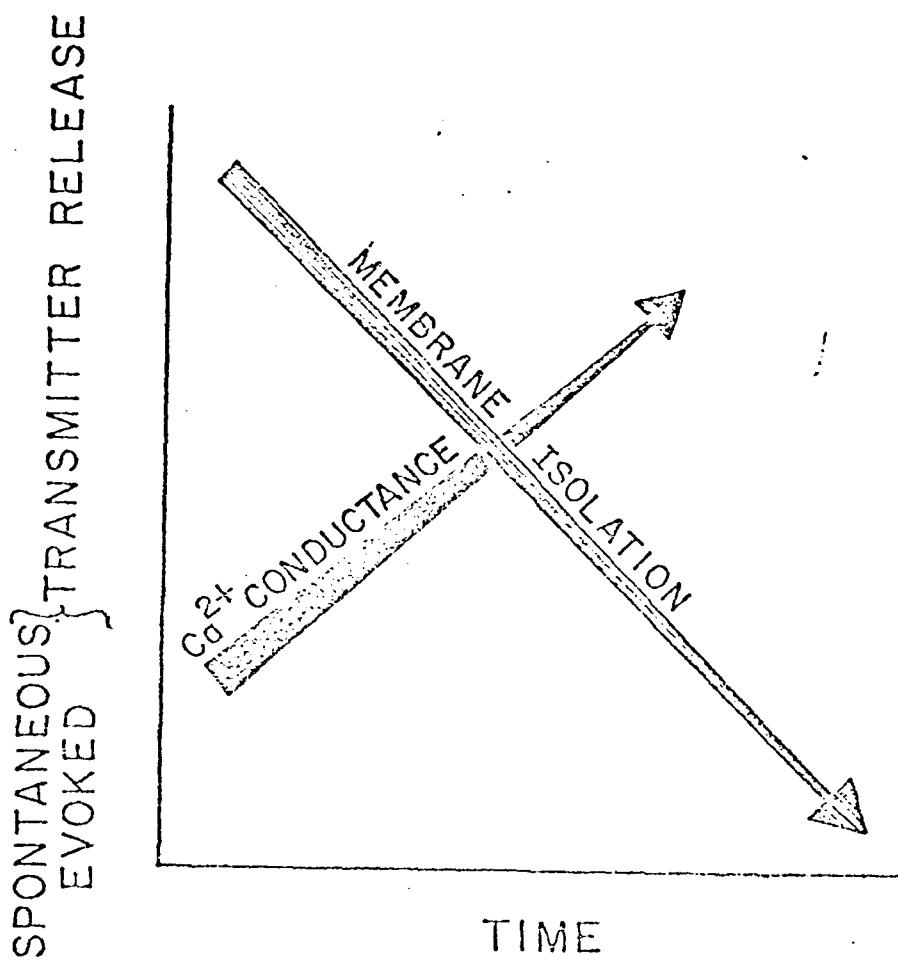
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Figure 6



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ABSTRACT

Some endotoxins produce a biphasic effect on spontaneous transmitter secretion, with an early facilitation of release, but a monotonic depression of evoked release triggered by nerve stimulation, while lipopolysaccharide produces only a monotonic depression of both spontaneous and evoked transmitter secretion under physiological conditions. However, glycolipid produces a biphasic effect on both spontaneous and evoked release. This suggests that the variety of effects seen in less pure preparations of glycolipids are modulations of the biphasic pattern of action resulting from differences in extraction procedures, presence of polysaccharide, or contamination of the specific endotoxin preparation employed. Based on these experiments we have proposed experimentally based mechanisms for the action of bacterial glycolipid: (1) an early, transient ionophoric action for calcium which facilitates secretion and (2) a late increasing isolation of the membrane from extracellular space.